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Identification of biomarkers to measure HIV-specific mucosal and systemic CD8$^+$ T-cell immunity using single cell Fluidigm 48.48 Dynamic arrays

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ABSTRACT

Thirty genes composed of cytokines, chemokines, granzymes, perforin and integrins were evaluated in gut and splenic $K^dGag_{197-205}$-specific single CD8$^+$ T cells using Fluidigm 48.48 Dynamic arrays, with the aim of identifying biomarkers to predict effective mucosal and systemic vaccine efficacy. The mRNA expression profiles were analyzed in three ways: (i) the "number" of $K^dGag_{197-205}$-specific CD8$^+$ T cells expressing the biomarker, (ii) "level" of mRNA expression using principal component analysis (PCA) and (iii) poly-functionality in relation to RANTES expression. In total, 21 genes were found to be differentially expressed between the vaccine groups and the immune compartments tested. Overall, the PCA indicated that IL-13Rα2 or IL-4R antagonist adjuvanted vaccines that previously induced high-avidity mucosal/systemic CD8$^+$ T cells with better protective efficacy, the "level" of mRNA expression, specifically RANTES, MIP-1β, and integrin α4 in gut $K^dGag_{197-205}$-specific single CD8$^+$ T cells, were significantly elevated compared to unadjuvanted vaccine. Furthermore, significantly elevated granzymes/perforin levels were detected in IL-13$^{-/-}$ mice given the unadjuvanted vaccine, indicating that the degree of IL-13 inhibition (total, transient or no inhibition) can considerably alter the level of T-cell activity/poly-functionality. When splenic- and gut-$K^dGag_{197-205}$-specific CD8$^+$ T cells were compared, PC1 vs. PC2 scores revealed that not only RANTES, MIP-1β, and integrin α4 mRNA, but also perforin, granzymes A/B, and integrins β1 and β2 mRNA were elevated in spleen. Collectively, data suggest that RANTES, MIP-1β, perforin, and integrins α4, β1 and β7 mRNA in single HIV-specific CD8$^+$ T cells could be used as a measure of effective mucosal and systemic vaccine efficacy.

1. Introduction

Unlike mucosal vaccination, systemic vaccination induces CD8$^+$ T cell that home poorly to the mucosae (e.g. the gut, genito-rectal or the vaginal mucosae) [1–5]. Mucosal-associated lymphoid tissues are linked to what is known as the "common mucosal immune system", whereby antigenic stimulation at one site can result in immune responses in both local and distant mucosae, as a result of mucosal homing [6,7]. Homing of lymphocytes to specific tissues is driven by the expression of cell-surface adhesion molecules known as integrins or chemokine receptors. Integrins are composed of non-covalently linked α and β subunits [8] that have the ability to pair differentially to induce unique functions. α4β7 interacts with mucosal addressin cell adhesion molecule-1 (MAdCAM-1) expressed on capillary endothelial cells in the gastrointestinal mucosae [9] and αEβ7 interacts with E-cadherin expressed on intestinal epithelial cells [10]. Both these molecules can provoke lymphocyte homing to the gut mucosae [11–13]. Similarly, chemokine receptor CCR9 promotes cell homing to the small intestine by interacting with its receptor CCL25 (TECK), present on

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the intestinal epithelium. α4β1 interacts with vascular cell adhesion molecule-l (VCAM-1) and fibronectin [14,15]. α4β1-VCAM-1 binding can mediate firm adhesion of lymphocytes to the endothelium during blood cell extravasation and there is evidence that β1 integrins can also mediate leukocyte retention, T-cell activation and homing to lung mucosae [16,17]. Similarly, β2 integrins consisting of αLβ2 (CD11a/CD18 or LFA-1), αMβ2 (CD11b/CD18, Mac-1, CR3), αXβ2 (CD11c/CD18, p150, 95, CR4) and αDβ2 (CD11d/CD18) interact with intercellular adhesion molecule-1 (ICAM-1) and induce leukocyte adhesion, T-cell activation and homing to inflamed skin.

Until recently, IFN-γ production has been the main indicator of protective efficacy following vaccination, but many studies have now shown that IFN-γ expression alone does not always correlate with high avidity/quality $CD8^+$ T cells and protective efficacy [2,18–22]. In recent studies, elevated numbers of high-avidity polyfunctional HIV-specific T cells have been detected in HIV elite controllers compared to non-controllers [23–25], highlighting the importance of poly-functional $CD8^+$ T cell immunity. In different studies, RANTES, MIP-1α and MIP-1β of the natural ligands for chemokine receptor 5 (CCR5) plus perforin and granzymes have been implicated in HIV-1 protective efficacy [26–31].

In the context of an HIV-1 vaccination, the induction of effective cytotoxic $CD8^+$ T cells that migrate to the mucosae is thought to be of great importance in curtailing the infection. It is now well established that efficacy of a mucosal vaccine has to be assessed at the mucosae, not in the blood compartment. However, identifying markers that correlate with effective mucosal immunity has been a challenging task, which has hampered the design of effective mucosal vaccines for many mucosal pathogens. Specifically, difficulties associated with obtaining enough cells from mucosal tissue (e.g. gut, rectal, vaginal biopsies) have complicated the use of conventional techniques such as ELISpot and intracellular cytokine staining for measuring mucosal immunity [32]. Recently, the development of microfluidic devices (e.g. Fluidigm's integrated fluidic circuits and dynamic arrays) has enabled the measurement of multiple genes expression in antigen-specific single cells [33].

Surprisingly, no study to date has evaluated a comprehensive panel of integrins following mucosal HIV vaccination, especially at the single cell level. Thus, in this study, following intranasal/intramuscular HIV prime-boost vaccination, $K^dGag_{197–205}$-specific Peyer's patch and splenic single $CD8^+$ T cells were sorted and the expression profiles of 30 different mRNAs composed of cytokines, chemokines, integrins, perforin and granzymes were analyzed. The mRNA expression profiles were evaluated in four vaccine conditions (including IL-13Rα2 and IL-4R antagonist adjuvanted vaccines that have shown to induce high-avidity CD8 T cells [34,35]) using Fluidigm 48.48 Dynamic array with the aim of identifying surrogate mucosal-specific biomarkers that would effectively measure mucosal immunity.

2. Materials and methods

2.1. Immunization of mice and mucosal/systemic lymphocytes preparation

Pathogen-free 6–8-week-old female BALB/c WT and BALB/c IL-13 KO mice were prime-boost immunized with i.n. FPV-HIV/i.m.VV-HIV unaduvanted vaccine strategy or IL-13Rα2-adjuvant or IL-4R antagonist adjuvant vaccine strategies as in Table 1 [34,35], under isoflurane anesthesia two weeks apart similar to our previous studies [2,22,34,35]. To measure systemic and mucosal immune responses, mice were euthanized at 2 weeks post-booster vaccination; spleen and Peyer's patches were removed and single cell suspensions were prepared as described previously [35,36].

Table 1
Mice and prime-boost vaccine strategies used in this study.

	Mice	Prime	Boost
1	BALB/c	i.n. FPV-HIV	i.m. VV-HIV
2	IL-$13^{-/-*}$	i.n. FPV-HIV	i.m. VV-HIV
3	BALB/c	i.n. FPV-HIV-IL-13Rα2Δ10**	i.m. VV-HIV-IL-13Rα2Δ10**
4	BALB/c	i.n. FPV- HIV-IL-4C118**	i.m. VV- HIV-IL-4C118**

All recombinant fowlpox virus (rFPV) and recombinant vaccinia virus (rVV) constructs encode HIV-1 gag/pol antigens. i.n. intranasal, i.m. intramuscular. The i.n. rFPV was given in a final volume of 20–25 μl, where i.m. rVV immunization was delivered in a 100 μl volume. All viruses were diluted in sterile phosphate-buffered saline (PBS) and then sonicated, to obtain homogeneous viral suspensions before delivery.

Note. In previous studies *IL-$13^{-/-}$ mice given the control unadjuvanted vaccine strategy [22] and **BALB/c mice given the IL-13Rα2 adjuvanted (IL-13Rα2Δ10) or IL-4R antagonist adjuvanted (IL-4C118) HIV vaccines that transiently inhibit IL-4 and/or IL-13 were found to induce mucosal and systemic $K^dGag_{197–205}$-specific $CD8^+$ T cells of higher avidity which was associated with greater cell mediated protective efficacy compared to BALB/c mice given the control unadjuvated vaccine [34,35] (avidity profiles were IL-$13^{-/-}$ unaduvanted > BALB/c adjuvanted > BALB/c unadjuvanted).

2.2. Tetramer and homing markers staining

Allophycocyanin (APC) conjugated $K^dGag_{197–205}$ tetramers were synthesized at the Bio-Molecular Resource Facility at JCSMR/ANU. Tetramer staining was performed as described previously [2,37]. Briefly, 2×10^6 splenocytes and mucosal lymphocytes were stained with anti-CD8-FITC antibody (BD PharMigen, San Diego, CA) and APC conjugated $K^dGag_{197–205}$ tetramer at room temperature for 40 min. To evaluate homing markers by flow cytometry (FACS), following tetramer staining cells were also surface stained with biotin labeled anti-mouse α4β7 (e-Biosciences, USA), PE conjugated anti-mouse αLβ2 (Biolegend, USA), FITC conjugated anti-mouse CCR9 (Biolegend, USA) and/or anti-mouse CD29/β1 (Biolegend, USA) antibodies for 25 min at 4 °C, followed by brilliant violet 421 streptavidin (Biolegend, USA) for 25 min at 4 °C. Cells were fixed and total 10^6 events per sample were collected using Fortessa flow cytometer (Becton-Dickinson), and results were analyzed using Cell Quest Pro software.

2.3. Evaluation of single-cell mRNA expression profiles using Fluidigm 48.48 Dynamic arrays

$K^dGag_{197–205}$ tetramer$^+$ $CD8^+$ single cells from spleen and/Peyer's patches were sorted[1] using cell FACS Aria I (BD Biosciences) into 96-well plates containing CellsDirect qRT-PCR reaction buffer (Invitrogen), Platinum Taq polymerase/SuperScript III reverse transcriptase (Invitrogen), a mixture of Taqman primer-probes at 0.2× concentration specific for the transcripts of interest, as listed in Supplementary Table 1 (Applied Biosystems), and SuperaseIn RNase inhibitor. Immediately following cell sorting, samples were centrifuged, and subjected to 20 cycles of pre-amplification using polymerase chain reaction (PCR) (cell cycling as: 1× 50 °C 15 min for the reverse transcription, 1× 95 °C 2 min for reverse transcriptase inactivation and Taq polymerase activation, followed by 20 cycles 95 °C 15 s and 60 °C 4 min amplification cycle). Subsequently, the pre-amplified single-cell cDNA was stored at −20 °C until analysis and was diluted with dH_2O (1:2) prior to qPCR reaction. Each pre-amplified cDNA sample was then separated into 48 separate reactions for qPCR analysis using the

[1] NOTE: The purity of these cells was 99% as stringent gating was used to determine $CD8^+$ tetramer$^+$ cells as done in our previous studies in spleen [2,35] and Peyer's patches [35,68]. Also, non HIV-specific tetramer is used as a negative control to clearly established that the double positive cells detected are true HIV-specific CD8 T cells.

BioMark 48.48 Dynamic array nanofluidic chip (Fluidigm Inc., USA) according to manufacturer's instruction (see Supplementary Fluidigm section for chip loading).

2.4. Statistical analysis

Amplification signals were analyzed and C_t values were determined using Fluidigm's real-time PCR analysis software version 4. In a preliminary analysis, C_t values were dichotomised based on the presence/absence of gene expression and the number of cells expressing a particular gene [38] in the groups indicated in Supplementary Table 1 were then compared using Fisher's exact test with SPSS (Statistical Package for the Social Sciences, International Business Machine Corp., USA) version 21.0 [39]. Principal components analysis (PCA) was used to assess patterns of mRNA expression levels of genes, and expression level/polyfunctionality was analyzed using a Matlab software package [40] (see Supplementary information section for more details). FACS data the standard error of the mean and p values were determined using one-way analysis of variance followed by Bonferroni's post tests for comparison of selected pairs in GraphPad Prism software version 5.

3. Results

3.1. IL-13Rα2 and IL-4R antagonist adjuvanted HIV vaccines can induce distinct mRNA expression profiles in gut-$K^dGag_{197–205}$-specific $CD8^+$ T cells

The gut-$K^dGag_{197–205}$-specific single cells obtained from novel IL-13Rα2 and IL-4R antagonist adjuvanted HIV vaccinated groups showed distinct mRNA expression profiles (Supplementary Fig. 1c and d) compared to the WT or IL-13 KO animals that received the standard unadjuvanted vaccination (Supplementary Fig. 1a and b). Unlike cytokine expression, selected chemokines/their receptors, perforin, granzymes and integrin expression patterns were significantly different between the four vaccination groups tested.

Firstly, when quantitative analysis was performed as a percentage of single gut $K^dGag_{197–205}$-specific $CD8^+$ T cells expressing the mRNAs of interest, at the chemokine level mRNA expression hierarchy of RANTES > MIP-1β > MIP-1α was detected between the four vaccination groups tested (Fig. 1a). The number of RANTES expressing gut $K^dGag_{197–205}$-specific $CD8^+$ cells was significantly higher in the IL-4R antagonist adjuvanted vaccinated group (83%) and the IL-13 KO mice (89%) given the unadjuvanted vaccine compared to the WT BALB/c mice (66%) (unadjuvanted vaccine vs. IL-13 KO $p = 0.008$; unadjuvanted vaccine vs. IL-4R antagonist vaccine $p = 0.016$) (Fig. 1a). The CCR5 also displayed a similar expression profile to RANTES where unadjuvanted vaccine vs. IL-13 KO $p = 0.001$; unadjuvanted vaccine vs. IL-4R antagonist vaccine $p = 0.019$ (Fig. 1a). Even though no statistical significance was observed, IL-13Rα2 adjuvanted group also showed greater increase in both RANTES and CCR5 compared to the control unadjuvanted group.

Next, when the numbers of granzymes and perforin expressing cells (critical mediators of anti-viral cytotoxic immunity) were evaluated, 10–40% gut $K^dGag_{197–205}$-specific $CD8^+$ T cells were found to express granzymes A and B, while 60–80% expressed perforin, and no granzyme C was detected in all four groups tested, where the expression hierarchy was perforin > granzymes A and B (Fig. 1b). Interestingly, the perforin mRNA expression profile was significantly different between WT mice and IL-13 KO mice that received the unadjuvanted vaccine ($p = 0.009$) unlike the group that received the adjuvanted vaccines (Fig. 1b).

Unlike transient inhibition of IL-13 (IL-13Rα2 adjuvanted vaccine), the complete absence of IL-13 (e.g. IL-13 KO mice) significantly reduced the number of gut $K^dGag_{197–205}$-specific $CD8^+$ T cells expressing the memory differentiation marker CD62L (L-selectin/Sell) (WT vs. IL-13 KO $^*p = 0.020$) and activation marker CD69 (WT vs. IL-13 KO $^{**}p = 0.002$) (Fig. 1c), but enhanced expression of P-selectin ligand or Selplg (WT vs. IL-13 KO $^*p = 0.017$) were detected. No significant differences in CD62L, P-selectin ligand, CCR7 or CD69 mRNA expression were found in BALB/c mice given the unadjuvanted or adjuvanted vaccines (Fig. 1c).

3.2. IL-13 inhibition can induce distinct integrin mRNA expression profiles in gut-$K^dGag_{197–205}$-specific $CD8^+$ T cells

Since an effective vaccine against HIV-1 should have the ability to recruit antigen-specific effector $CD8^+$ T cells to the mucosae, next the integrin expression profiles were evaluated. Knowing that α4 can heterodimerize with either β7 or β1, while αE can only heterodimerize with β7, when these integrins were evaluated in gut $K^dGag_{197–205}$-specific $CD8^+$ cells, α4 and β7 mRNA were detected in 60–90% of the cells from all groups tested (Fig. 1d). β1 mRNA expression was similar between the BALB/c mice that received the unadjuvanted or adjuvanted vaccines (~40%) (Fig. 1d). However, the IL-13 KO animals that received the unadjuvanted vaccination showed significantly elevated numbers of cells expressing α4 and β1 mRNA (WT vs. IL-13 KO, $^*p = 0.014$; WT vs. IL-13 KO, $^*p = 0.017$) and significantly reduced numbers of cells expressing αE were detected (WT vs. IL-13 KO, $^*p = 0.012$) compared to the wild type animals that received the same vaccine (Fig. 1d).

β2 integrin is known to heterodimerize with αD, αL, αM or αX. Our data revealed that out of these integrins, only αL and αX mRNA were detected in gut $K^dGag_{197–205}$-specific $CD8^+$ T cells (Fig. 1e). The number of gut $K^dGag_{197–205}$-specific $CD8^+$ T cells that expressed αL and β2 mRNA was significantly lower in IL-4R antagonist adjuvanted group compared to the BALB/c mice that received the unadjuvanted vaccine ($^*p = 0.03$) (Fig. 1e). Also very low numbers of cells were found to express in αX mRNA in all groups tested with no significant difference.

3.3. Quantitatively distinct mRNA expression profiles were observed following novel adjuvanted vaccination compared to unadjuvanted control vaccine strategy

To establish the qualitative differences in log mRNA expression and to identify potential mucosal-specific $CD8^+$ T-cell biomarkers, next the mRNA expression levels in single gut-$K^dGag_{197–205}$-specific $CD8^+$ T cells were detected using a multivariate PCA approach (see Supplementary statistics section for details). In this analysis, the rank-correlated expression (Fig. 2a) of 21 mRNAs was best approximated by the four principal components (PC1, PC2, PC3 and PC4) that explained at least 75% of the total variance.

The first principal component (PC1) explained 36% of the total variance in overall log expression. This axis reflected the pattern of positively correlated expression of RANTES, MIP1β, perforin, CCR5 and integrin α4 and negatively correlated expression of CCR9, CCR7 and Sell/L-selectin (Fig. 2b). PC1 scores differentiated the adjuvanted vaccine groups which had higher relative expression of RANTES, MIP1β, and integrin α4 and correspondingly lower relative expression of CCR9, CCR7, Sell/L-selectin and integrin αE, from the unadjuvanted vaccine group ($p < 0.001$) (Fig. 2b–d).

The second principal component (PC2) explained 18% of the total variation in overall log expression. This axis reflected the coordinated expression of MIP1α, MIP1β, RANTES, CCR5, granzymes A and B, TNF-α and IFN-γ (Fig. 2b). Mean PC2 scores differed significantly between the IL-13 KO and other vaccine groups with IL-13 KO mice showing increased expression patterns of MIP1α, MIP1β,

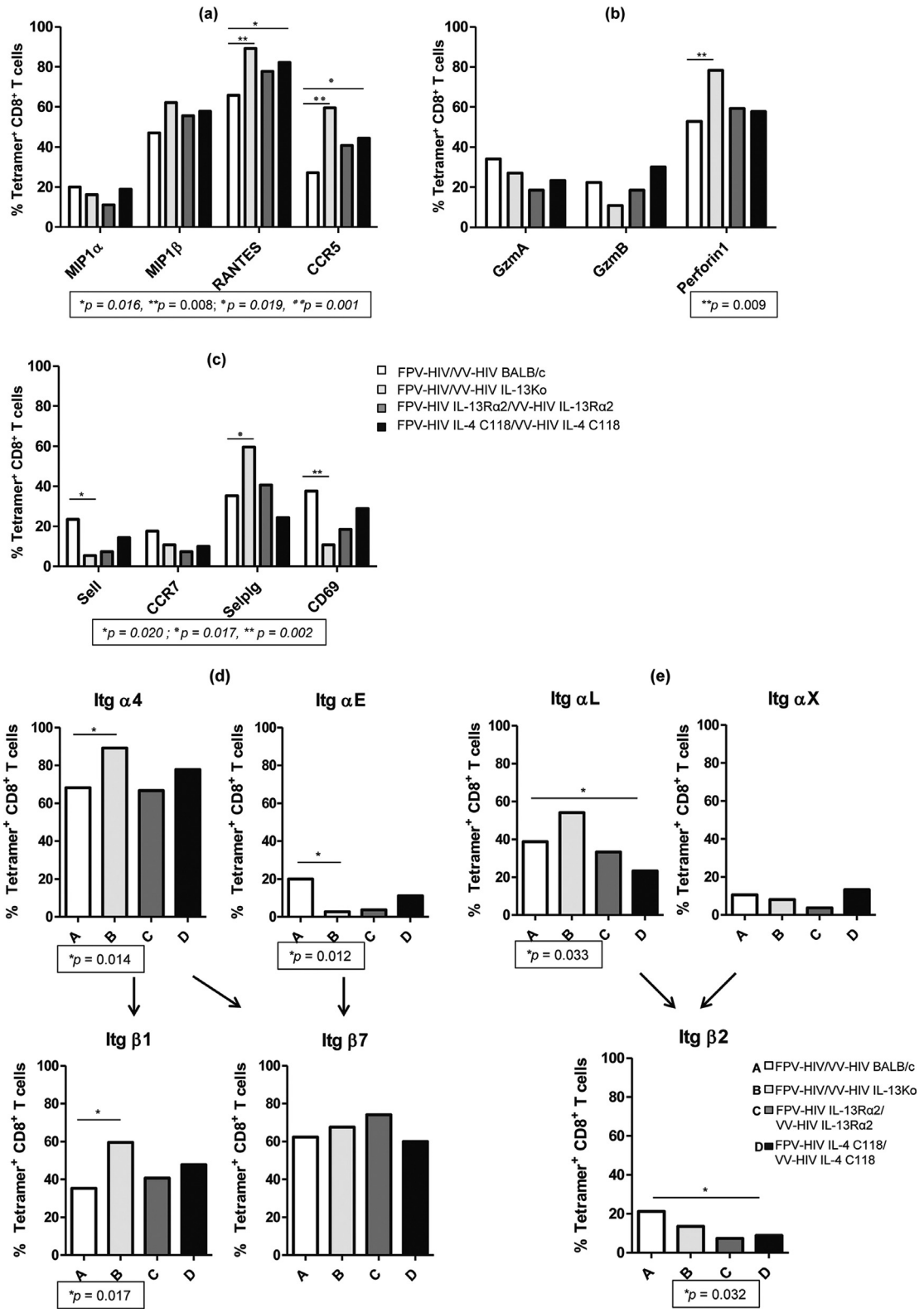

Fig. 1. Detailed evaluation of percentage of mucosal $K^dGag_{197-205}$ tetramer$^+$ CD8$^+$ single cells expressing particular gene in different vaccine groups compared to the standard FPV-HIV/VV-HIV vaccine. Wild type (WT) BALB/c and IL-13 gene knock out (KO) mice ($n = 4–5$) were immunized (i.n./i.m.) with vaccines indicated in Table 1. Fourteen days post boost immunization tetramer staining of Peyer's patch samples was performed, $K^dGag_{197-205}$-specific CD8$^+$ single cells were sorted into 96-well plate (total 48 cells per group), and cDNA was synthesized followed by single cell gene expression analysis using Fluidigm's Biomark™ real-time PCR system (Supplementary Table 1). The graphs represent the percentages of $K^dGag_{197-205}$ tetramer$^+$ CD8$^+$ single cells (*y*-axis) in Peyer's patches expressing MIP1α, MIP1β, RANTES and CCR5 (a), granzyme A, granzyme B, perforin1 (b), sell/CD62L, CCR7, selplg/P-selectin ligand and CD69 (c). The graph also shows percentage of $K^dGag_{197-205}$-specific CD8$^+$ T cells expressing integrins (d, e) in different vaccine groups. The *p* values were determined using Fisher's exact statistical test (note: $^*p$ values in the box are for genes mentioned left to right on *x*-axis). Data represent two experiments for standard unadjuvanted vaccine and IL-4R antagonist adjuvanted vaccine and one experiment for IL-13 KO and IL-13Rα2 adjuvant vaccine group. Note: in this study, where less than 10% of the single cells expressed the mRNA of interest, they were excluded from further analysis (example: IL-2, IL-4, IL-13, IL-17, CCR10, granzyme C, and integrins αM and αD).

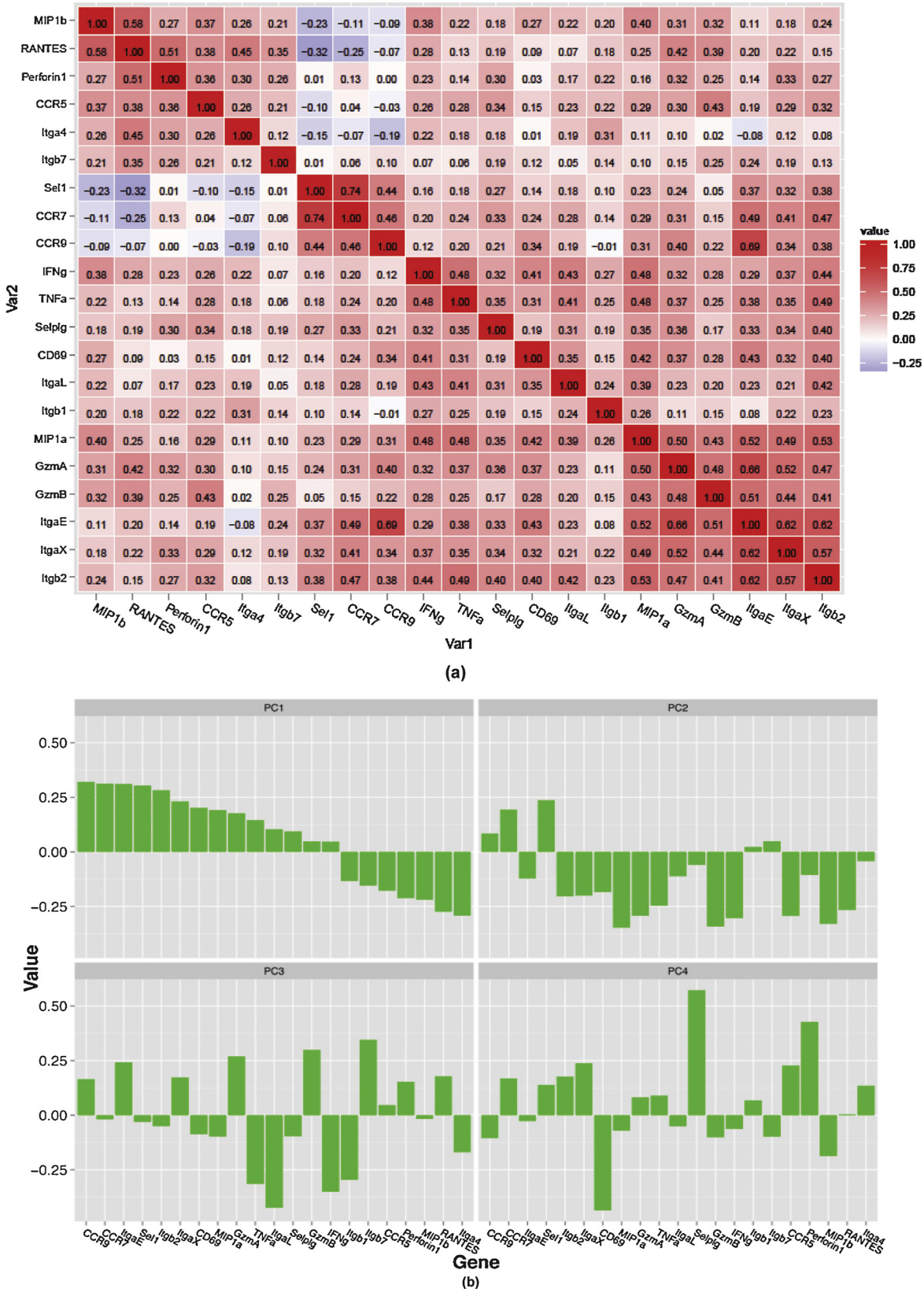

Fig. 2. PCA of $K^dGag_{197-205}$-specific single $CD8^+$ T cell gene expression patterns in Peyer's patches following different vaccinations. The rank-correlated expression of 21 mRNA's (a) and the genes contribution in each principle component are shown (b). The co-expression levels of gene transcripts within single $K^dGag_{197-205}$-specific $CD8^+$ T cell were analyzed in PC1 vs. PC2 component (c), PC1 vs. PC4 component (d) and PC1 vs. PC3 component (e). In each PCA plot the dot represents $K^dGag_{197-205}$-specific single $CD8^+$ T cell (red: FPV-HIV IL-4C118/VV-HIV IL-4C118, $n = 86/96$ single cells; green: FPV-HIV IL-13Rα2/VV-HIV IL-13Rα2, $n = 27/48$ single cells; blue: FPV-HIV/VV-HIV IL-13 KO, $n = 37/48$ single cells; black: FPV-HIV/VV-HIV BALB/c, $n = 83/96$ single cells). The bold bigger dots represent mean gene expression level for different vaccine groups tested; analysis of variance (ANOVA) was used to compare the primary principal component scores across the groups as mentioned in Section 2. Data represent two experiments for standard unadjuvanted vaccine and IL-4R antagonist adjuvanted vaccine and one experiment for IL-13 KO and IL-13Rα2 adjuvant vaccine group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

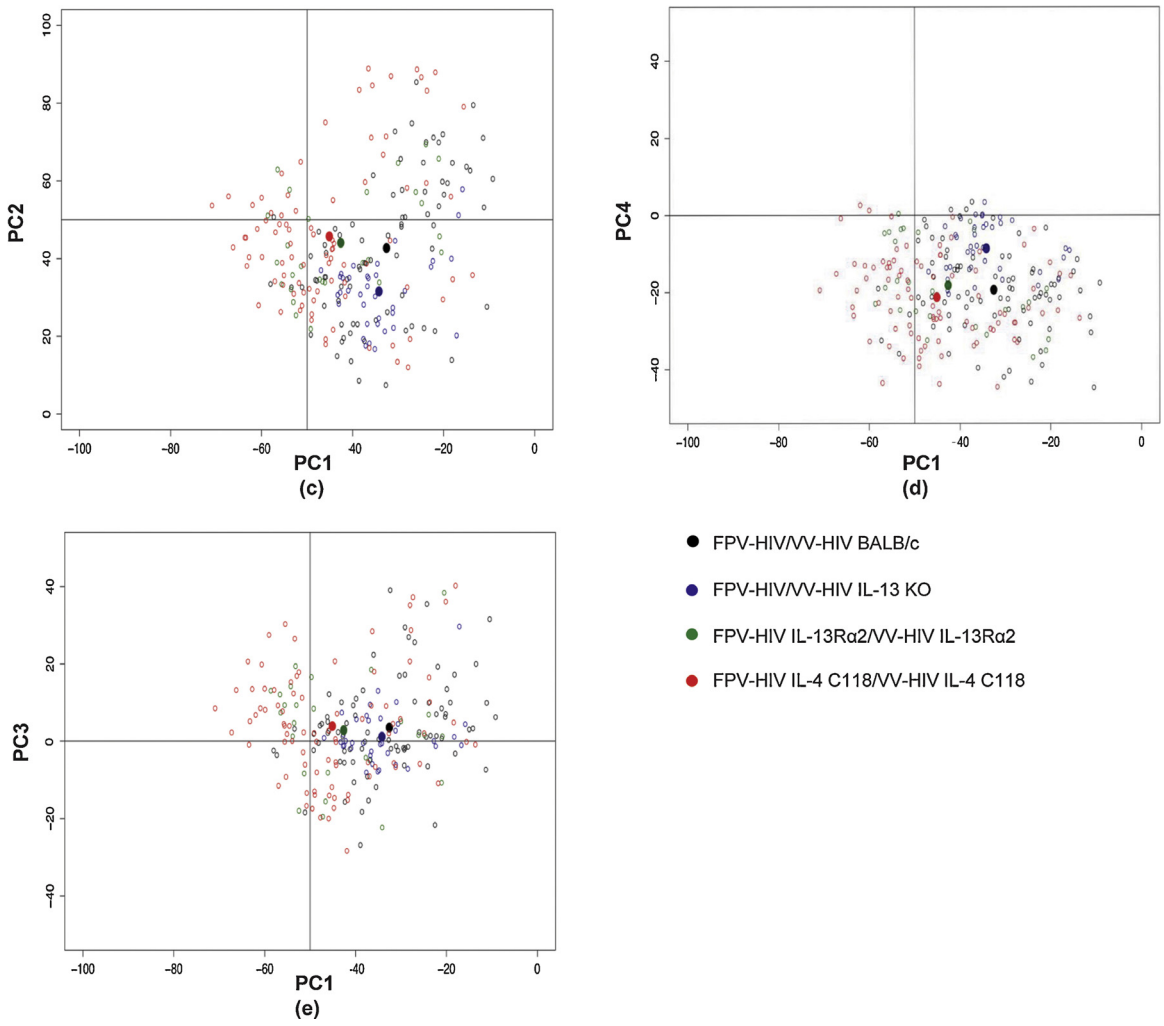

Fig. 2. (*Continued*).

RANTES, CCR5, granzymes A and B, TNF-α and IFN-γ compared with the other groups ($p < 0.001$) (Fig. 2c). The fourth principal component (PC4) likewise described significant differences between IL-13 KO and other vaccine groups tested and corresponded to relative low expression of CD69 and high expression of P-selectin ligand (Selplg) and perforin mRNA in IL-13 KO vaccinated group ($p < 0.01$) (Fig. 2b and d). The third principal component (PC3) corresponded with no significant differences between all the groups tested (Fig. 2e).

Overall, our PCA results indicated that (i) following novel IL-13Rα2 or IL-4R antagonist adjuvanted vaccination, the "level" of mRNA expression (specifically RANTES, MIP-1β, and integrin α4) in gut $K^dGag_{197-205}$-specific single $CD8^+$ T cells was significantly elevated compared to unadjuvanted control vaccine and (ii) the mRNA expression patterns in animals which received IL-13Rα2 or IL-4R antagonist adjuvanted vaccines showed significantly different mRNA expression profiles to that of IL-13 KO mice.

3.4. Distinct mRNA expression profiles were detected in systemic and mucosal $K^dGag_{197-205}$-specific single $CD8^+$ T cells

With the goal of identifying possible surrogate markers of effective mucosal immunity in the systemic compartment, next splenic and Peyer's patch $K^dGag_{197-205}$-specific single $CD8^+$ T cells were analyzed between unadjuvanted and IL-4R antagonist vaccinated groups. Data revealed that the numbers of $K^dGag_{197-205}$-specific $CD8^+$ T cells that expressed RANTES and granzyme A mRNA were significantly higher in spleen while CD62L and CD69 were significantly higher in Peyer's patch (Fig. 3a). Additionally, the IL-4R antagonist adjuvanted vaccinated group also showed significantly elevated numbers: $K^dGag_{197-205}$-specific $CD8^+$ T cells expressing perforin ($p = 0.03$) and MIP-1α mRNA ($p = 0.027$) in spleen and Peyer's Patch, respectively (Fig. 3a). Similarly, in the unadjuvanted vaccine group, Granzyme B ($p = 0.021$) and CCR7 ($p = 0.0001$) mRNA expression was significantly higher in gut $K^dGag_{197-205}$-specific $CD8^+$ T cells compared to spleen. Also in gut $CD62L^-$ $CCR7^-$ effector memory (Em) phenotype was greater (80%) than $CD62L^+$ $CCR7^+$ central memory (Cm) $K^dGag_{197-205}$-specific $CD8^+$ T-cell phenotype (20%), no Cm CD8 T cells were detected in spleen (Fig. 3a).

3.5. Distinct integrin/CCR9 mRNA and protein expression profiles were detected in systemic and mucosal $K^dGag_{197-205}$-specific single $CD8^+$ T cells

Knowing α4 can heterodimerize with β7 and β1 while αE can only heterodimerize with β7, next mRNA expression profiles of these integrins in spleen and Peyer's patch $K^dGag_{197-205}$-specific single $CD8^+$ T cells were evaluated. In both compartments, integrins α4 (80–90%) and β7 (50–70%) expressions were found to be similar following unadjuvanted and IL-4R antagonist adjuvanted vaccination (Fig. 3b). However, compared to Peyer's patch significant increase in the number of splenic $K^dGag_{197-205}$-specific single $CD8^+$

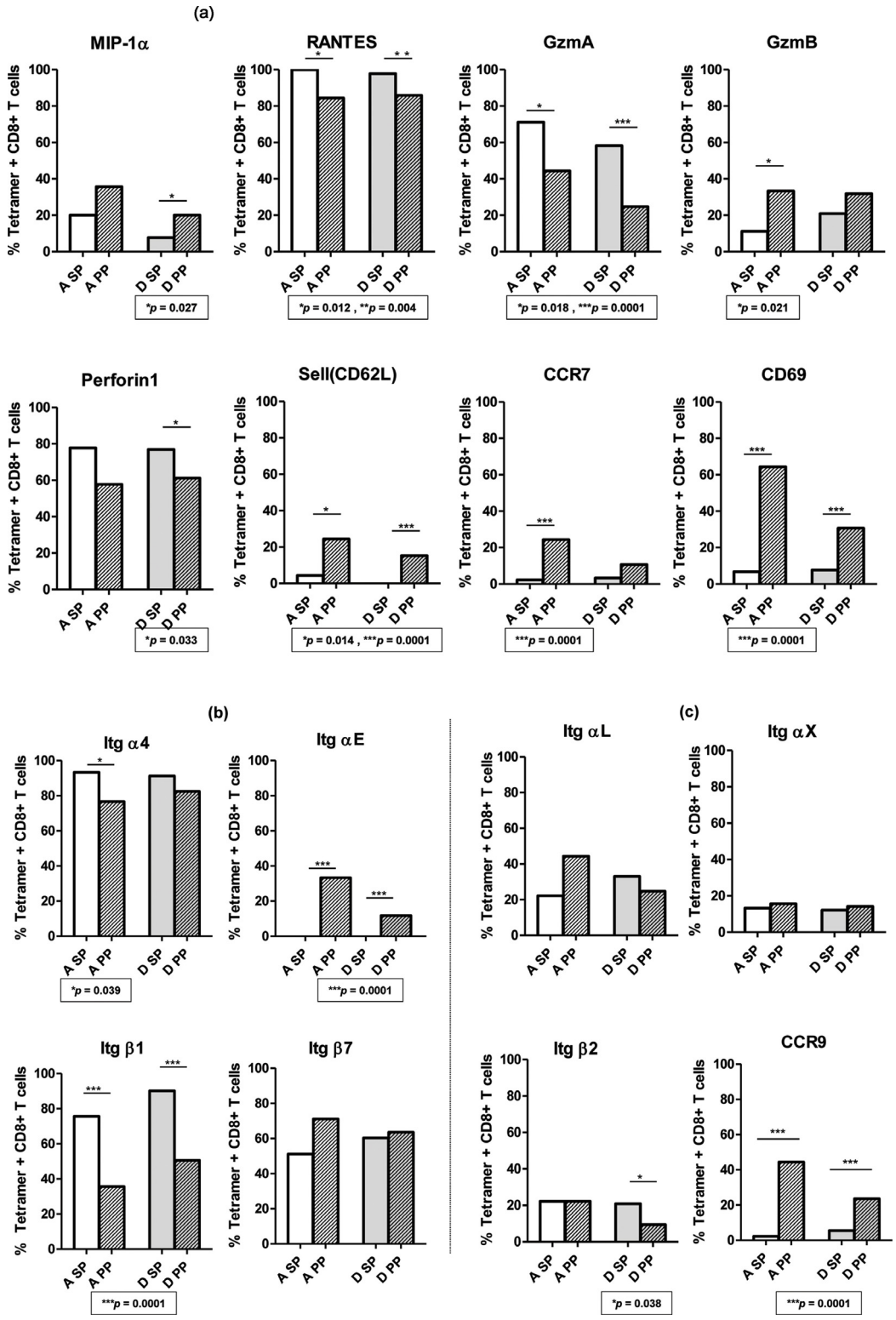

Fig. 3. Evaluation of percentage of $K^dGag_{197-205}$-specific single $CD8^+$ T cell expressing genes in systemic and mucosal compartments. BALB/c mice ($n = 5$ per group) were immunized i.n./i.m. with standard FPV-HIV/VV-HIV vaccine or FPV-HIV IL-4C118/VV-HIV IL-4C118 vaccine. Fourteen days post boost immunization, spleen and Peyer's patches were harvested and single-cell suspensions prepared; following tetramer staining $K^dGag_{197-205}$-specific single splenic and Peyer's patch $CD8^+$ T cells (total of 48 cells) were sorted from the same group of animals, cDNA was synthesized and mRNA expression was evaluated using Fluidigm Biomark™ real-time PCR system as mentioned in Section 2. The graphs (a–c) represent percentage of $K^dGag_{197-205}$-specific single $CD8^+$ T cell expressing indicated genes, the *x*-axis A SP and A PP = spleen and Peyer's patches of FPV-HIV/VV-HIV vaccinated group, D SP and D PP (gray filled) = spleen and Peyer's patches of FPV-HIV IL-4C118/VV-HIV IL-4C118 vaccinated group. Following standard or novel vaccination, the percentages of tetramer-specific single $CD8^+$ T cells expressing particular gene were compared between spleen and Peyer's patches; significant differences as indicated by *p*-values below the graphs were determined using Fisher exact's test. Standard unadjuvanted vaccine represents one experiment and IL-4R antagonist adjuvanted vaccine represents data from two experiments. The spleen and Peyer's patches were harvested from same experiment and the statistical significance is shown between the two compartments within a vaccination group.

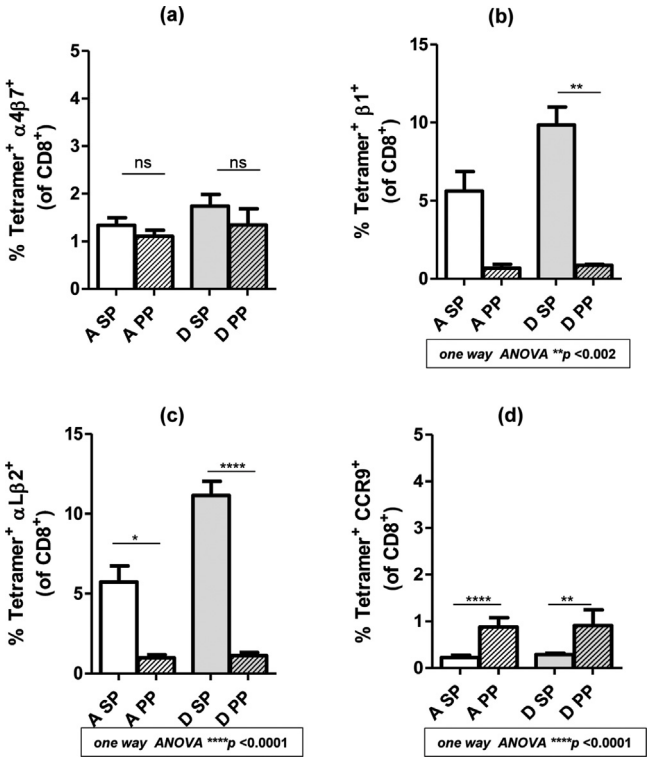

Fig. 4. Evaluation of integrin protein expression in $K^dGag_{197–205}$-specific $CD8^+$ T cells in spleen and/Peyer's patches using flow-cytometry. BALB/c mice ($n = 4$ per group) were immunized i.n./i.m. with standard unadjuvanted vaccine or IL-4R antagonist adjuvanted vaccine, 14 days post boost immunization spleen and Peyer's patches were collected (note: Peyer's patches were pooled from all animals), tetramer and homing marker staining was performed as mentioned in Section 2, and the percentages of $K^dGag_{197–205}$-specific $CD8^+$ T cells that expressed integrin α4β7 (a), β1 (b), αLβ2 (c), and CCR9 (d) were analyzed in spleen and Peyer's patches using flow-cytometry. The *x*-axis A SP and A PP = spleen and Peyer's patches of FPV-HIV/VV-HIV vaccinated group, D SP and D PP (gray filled) = spleen and Peyer's patches of FPV-HIV IL-4C118/VV-HIV IL-4C118 vaccinated group, error bars indicate standard error of mean, the *p* values for multiple comparisons were obtained from one-way analysis of variance followed by Bonferroni's post hoc tests. Data for gut-specific homing markers expression α4β7 and CCR9 by tetramer$^+$ $CD8^+$ T cells represent average of 5–7 experiments and for integrins αLβ2 and β1 represent 2–3 experiments. Note: % $K^dGag_{197–205}$-specific $CD8^+$ T cells in spleen = 5–6% for standard FPV-HIV/VV-HIV vaccine and 10–12% for IL-4R antagonist vaccine; % $K^dGag_{197–205}$-specific $CD8^+$ T cells in Peyer's patches = 0.5–1.5% for standard FPV-HIV/VV-HIV vaccine and 2–3% for IL-4R antagonist vaccine consistent to our previous studies [34,35].

T cells that expressed integrin β1 mRNA was detected ($p < 0.0001$) (Fig. 3b) while, higher numbers of integrin αE mRNA expressing cells were detected in Peyer's patch compared to spleen ($p < 0.0001$) (Fig. 3b) in both vaccine groups tested.

We next assessed mRNA expression of αL and αX which are known to heterodiomerize with integrin β2. Although data revealed that αL and αX were not significantly different between spleen and Peyer's patch in both vaccine groups tested (Fig. 3c), compared to Peyer's patch there was a significant increase in the number of splenic $K^dGag_{197–205}$-specific single $CD8^+$ T cells that expressed integrin β2 mRNA in IL-4R antagonist vaccinated group ($p = 0.038$) (Fig. 3c). In addition to integrins, in both vaccine groups, $K^dGag_{197–205}$-specific $CD8^+$ T cells that expressed the CCR9 were significantly elevated in Peyer's patch compared to spleen ($p = 0.0001$) (Fig. 3c). Flow cytometry analysis of $K^dGag_{197–205}$-specific $CD8^+$ T cells revealed that similar to what was observed at the mRNA level (Fig. 3a), no significant differences in heterodimer α4β7 were detected between spleen and Peyer's patches in both vaccines tested (Fig. 4a). In the IL-4R antagonist vaccine group, the number $K^dGag_{197–205}$-specific $CD8^+$ T cells that expressed β1 were elevated in spleen compared to Peyer's patch (Fig. 4b). Moreover, although there was no significant difference in αL mRNA in spleen and Peyer's patch, the numbers of αLβ2 expressing splenic $K^dGag_{197–205}$-specific $CD8^+$ T cells were significantly elevated in both vaccine groups tested (Fig. 4c). Our data also indicated that unlike α4β7, the number of $K^dGag_{197–205}$-specific $CD8^+$ T cells that expressed CCR9 were significantly elevated in Peyer's patch compared to spleen at both the mRNA (Fig. 3c) and protein level (Fig. 4d), suggesting that CCR9 is more specific to gut. Collectively, the single-cell mRNA expression analysis and flow cytometry analysis clearly indicated that following vaccination distinct gene expression profiles can be observed in systemic and mucosal $K^dGag_{197–205}$-specific $CD8^+$ T cells.

3.6. PCA revealed distinct mRNA expression profiles between systemic and mucosal compartments

Next PCA was performed to evaluate the differences between spleen and Peyer's patch. In this analysis, the rank-correlated expression (Fig. 5a) of 21 mRNAs was best approximated by four principal components (PC1, PC2, PC3, PC4) that explained at least 71.5% of the total variance. The PC1 explained 38% of the total variance in overall log expression. This axis reflected the coordinated expression of perforin, integrin α4 and β1 and also of CD69, CCR9, MIP-1α, Sell/L-selectin and CCR7 mRNA (Fig. 5b). PC1 scores differentiated the spleen which had relatively higher expression of perforin, integrin α4 and β1 and lower expression of CD69, CCR9, MIP-1α, Sell/L-selectin and CCR7 from the Peyer's patches of respective vaccine groups ($p < 0.001$) (Fig. 5c). Mean PC1 scores also differentiated adjuvanted vaccine group which had relatively higher expression of perforin, integrin α4 and β1 in splenic $K^dGag_{197–205}$-specific $CD8^+$ T cells from the unadjuvanted group ($p < 0.01$) (Fig. 5c).

The PC2 explained 18% of the total variance in overall log expression. This axis reflected the coordinated mRNA expression profile of RANTES, MIP-1β, granzyme A and B and integrin β2 (Fig. 5b). Mean PC2 scores differed significantly between spleens and Peyer's patch with relatively higher expression of this profile in spleen ($p < 0.001$) (Fig. 5c). The PC3 explained only 8% of the total variance in overall log expression. Mean PC3 scores differed significantly between spleen and Peyer's patch and indicated relatively higher expression of integrin αL and lower expression of integrin β7 in spleen compared to Peyer's patches of respective vaccine groups (Fig. 5d). The PC4 corresponded with no significant differences between spleen and Peyer's patches (Fig. 5e).

3.7. The gut $K^dGag_{197–205}$-specific $CD8^+$ T cells that expressed RANTES were polyfunctional

As relatively higher proportion of gut $K^dGag_{197–205}$-specific $CD8^+$ T cells expressed RANTES in all the three groups when compared to the unadjuvanted vaccination (Fig. 1a), we next evaluated the probability of other mRNAs to co-express together with RANTES (polyfunctionality in relation to RANTES) using the Matlab script that was developed. Data indicated that more than 50% of RANTES expressing gut $K^dGag_{197–205}$-specific $CD8^+$ T cells obtained from (i) BALB/c mice given the unadjuvanted vaccine co-expressed MIP1-β, perforin, granzyme-A, integrin α4 and β7 (Fig. 6a), (ii) IL-13 KO mice given the unadjuvanted vaccine co-expressed MIP1-β, CCR5, perforin, P-selectin ligand, integrin α4, αL, β1 and β7 (Fig. 6b), (iii) FPV-HIV IL-13Rα2/VV-HIV IL-13Rα2 adjuvanted vaccinated group co-expressed MIP1-β, perforin, integrin α4 and β7 (Fig. 6c) and (iv) FPV-HIV IL-4C118/VV-HIV IL-4C118 adjuvanted vaccinated group co-expressed MIP1-β, perforin, CCR5, integrin α4, β1 and β7 (Fig. 6d). In FPV-HIV/VV-HIV and FPV-HIV IL-4C118/VV-HIV IL-4C118 vaccinated groups, $K^dGag_{197–205}$-specific splenic $CD8^+$ T

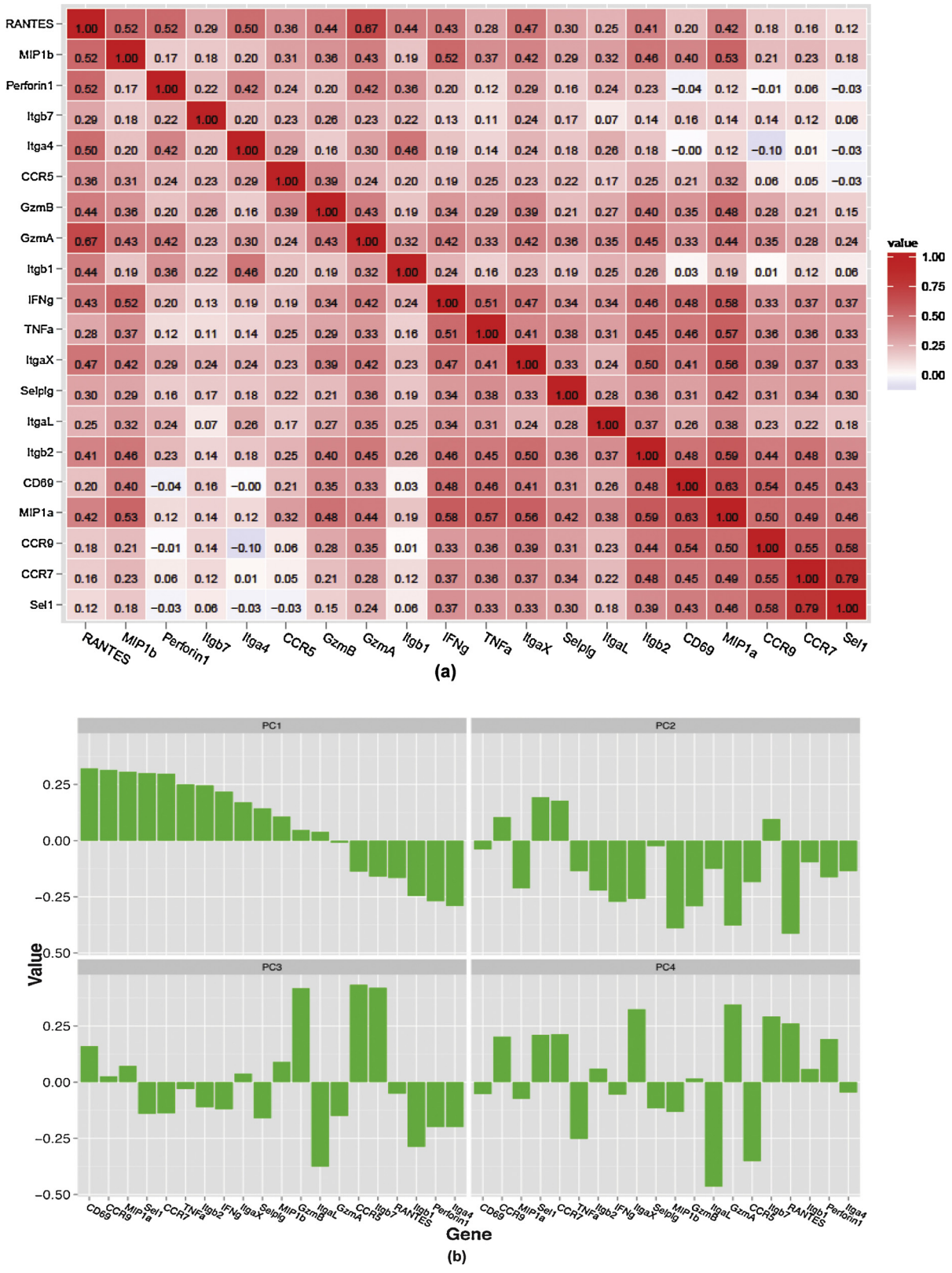

Fig. 5. PCA of $K^dGag_{197-205}$-specific single $CD8^+$ T-cell gene expression patterns in spleen vs. Peyer's patches. Following standard vaccine and/IL-4R antagonist adjuvanted vaccination mRNA expression levels were compared in between the two tissues. The rank-correlated expression of 21 mRNA's (a) and the genes contribution in each principle component are shown (b). In PC1 vs. PC2 plot (c), PC1 vs. PC3 plot (d) and PC1 vs. PC4 plot (e) the dots represent $K^dGag_{197-205}$-specific single $CD8^+$ T cells isolated from Peyer's patches and triangles represent $K^dGag_{197-205}$-specific single $CD8^+$ T cells isolated from spleen. The cluster of mean gene expression levels is indicated as filled black dot: Peyer's patch $K^dGag_{197-205}$-specific $CD8^+$ T-cell response after FPV-HIV/VV-HIV vaccination ($n = 45/48$ single cells); filled red dot: Peyer's patch $K^dGag_{197-205}$-specific $CD8^+$ T cell response after FPV-HIV IL-4C118/VV-HIV IL-4C118 vaccination ($n = 86/96$ single cells); filled blue triangle and yellow triangle for $K^dGag_{197-205}$-specific $CD8^+$ T cell response in spleen following FPV-HIV/VV-HIV ($n = 45/48$ single cells) and/FPV-HIV IL-4C118/VV-HIV IL-4C118 vaccination ($n = 91/96$ single cells), respectively. Data represent one experiment with standard vaccine and two experiments with IL-4R antagonist vaccine. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

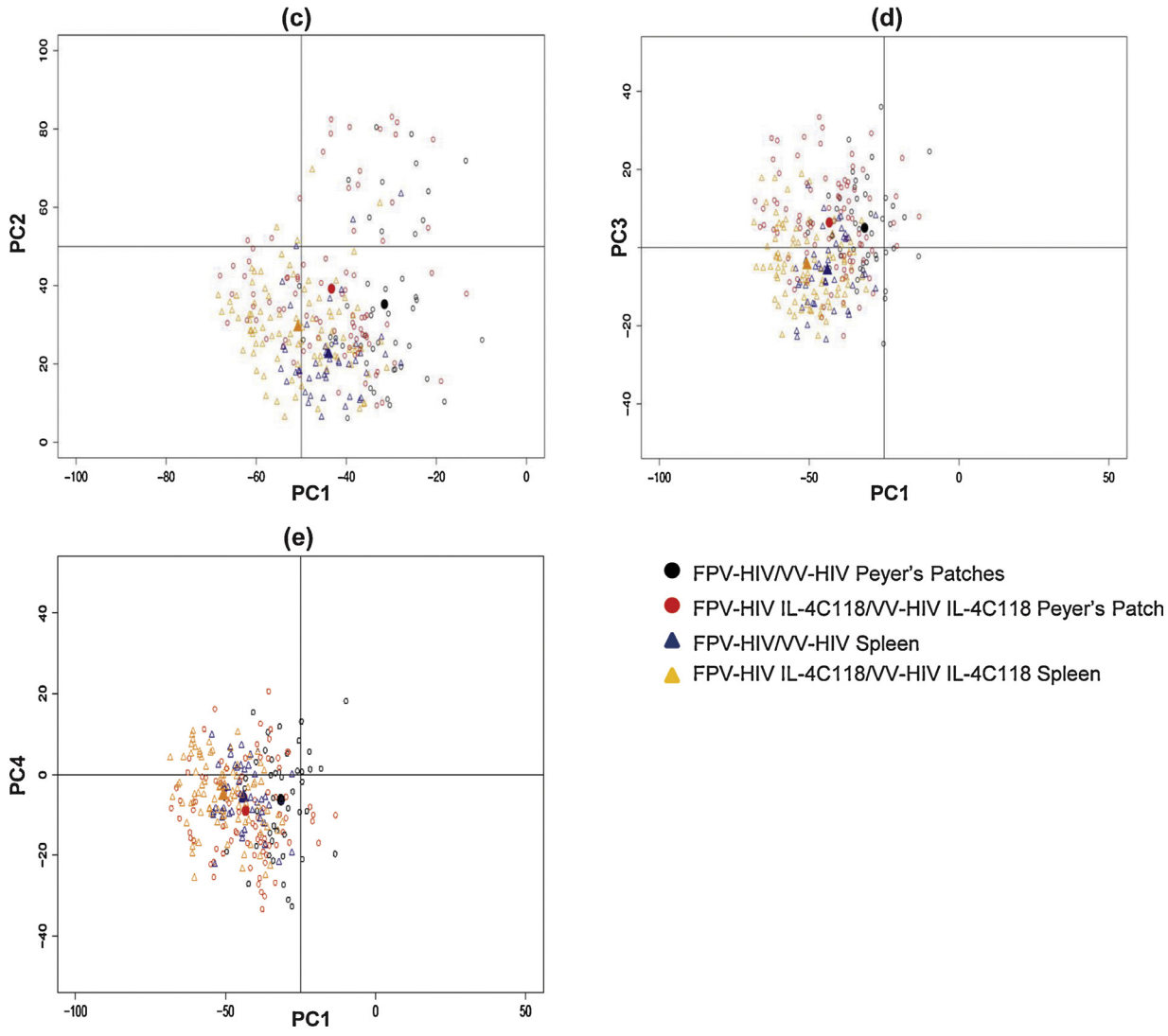

Fig. 5. (*Continued*).

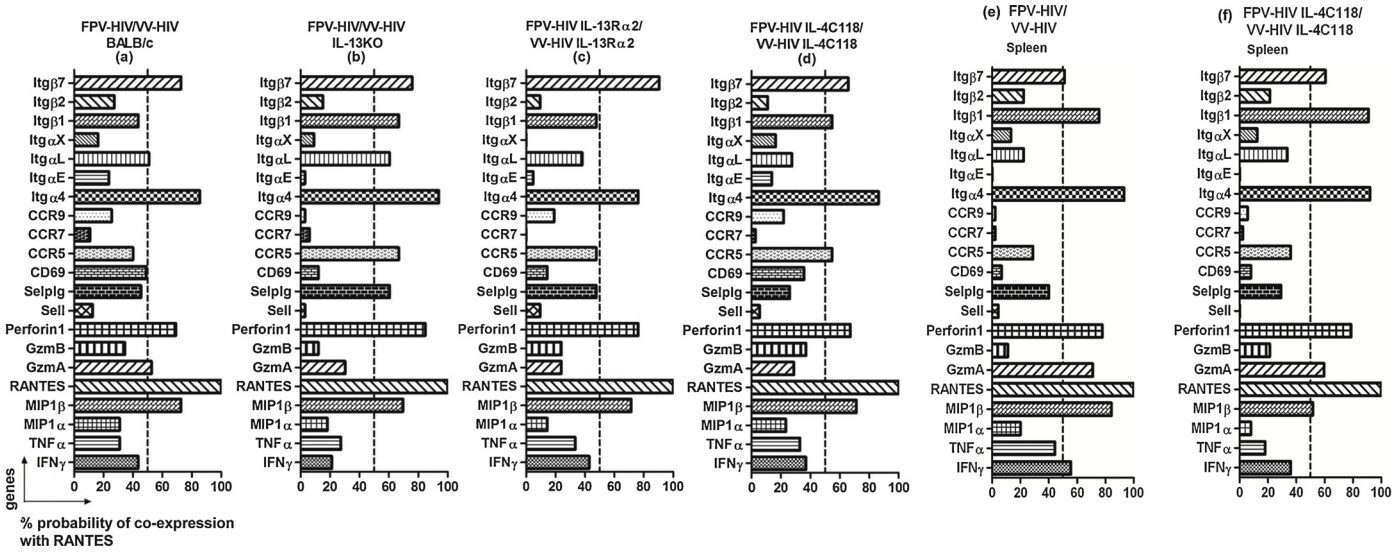

Fig. 6. Following different vaccinations, analysis of genes that were co-expressed with RANTES within $K^dGag_{197-205}$-specific single $CD8^+$ T cells. BALB/c and IL-13 KO mice were immunized i.n./i.m. with different vaccines as indicated (a–f), cells expressing RANTES were considered 100% and then the percentage probability of indicated genes co-expressed with RANTES in Peyer's patch (a–d) and spleen (e and f) were determined using Matlab software as indicated in Section 2. Data represent two experiments for standard unadjuvanted vaccine and IL-4R antagonist adjuvanted vaccine and one experiment for IL-13 KO and IL-13Rα2 adjuvant vaccine group. Note: Data presented in Figs. 1–6 were obtained from same vaccination experiment.

cells that expressed RANTES were also found to co-express MIP1-β, perforin, integrin α4 and β7 (Fig. 6e and f) and granzyme-A and integrin β1 were found to be greatly elevated in spleen compared to Peyer's patch (Fig. 6a, e and d, f).

Overall quantitative analysis of the data indicates that integrin αE (Fig. 3) and CCR9 (Figs. 3–6) were specific to Peyer's patch, and higher proportion and relative higher expression of granzyme A (Figs. 3–6) and integrin β2 (Figs. 3–5) were detected in spleen compared to Peyer's patches. However, RANTES, MIP1-β, perforin, integrin α4, β1 and β7 (Figs. 1–6) were found to be common biomarkers detected both in Peyer's patch and spleen (see Venn diagram Supplementary Fig. 2).

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4. Discussion

In the current study, 21 out of the 30 mRNAs tested were differentially expressed between unadjuvanted i.n. FPV-HIV/i.m. VV-HIV vaccine group compared to novel IL-13Rα2 and IL-4R antagonist adjuvanted vaccinated groups that have shown to induce high-avidity/poly-functional mucosal and systemic $K^dGag_{197-205}$-specific $CD8^+$ T-cell immunity [34,35]. The cytokine expression by gut or splenic-$K^dGag_{197-205}$-specific single $CD8^+$ T cells was not significantly different between the groups, which is not entirely surprising as unstimulated (no peptide/protein encountered) $K^dGag_{197-205}$-specific $CD8^+$ T cells have shown to express no or very low IFN-γ, TNF-α and IL-2 at the mRNA and proteins levels [2,22]. Multiple studies have shown that following vaccination measuring the expression of not only cytokines (i.e. IFN-γ) but also chemokines, granzymes and perforin is of great importance when evaluating vaccine efficacy [24,41]. The principal component analysis of gut-$K^dGag_{197-205}$-specific single cells revealed enhanced expression of RANTES and MIP-1β mRNA following IL-13Rα2 and IL-4R antagonist adjuvanted vaccination strategy including the IL-13KO mice given the unadjuvanted vaccine compared to the BALB/c unadjuvanted control. This is consistent with our previous finding where elevated RANTES was detected in IL-$13^{-/-}$ and IL-$4^{-/-}$ splenic $K^dGag_{197-205}$-specific $CD8^+$ T cells following unadjuvanted vaccination [22]. Macaques vaccinated with SHIV-4, the expression of RANTES together with IFN-γ, have been associated with protection against SIVsm challenge [26]. Involvement of RANTES in protection has also been reported with other chronic viral infections [42]. Moreover, in HIV non-progressors not only high levels of IFN-γ and [24] MIP-1β but also expression of perforin by HIV-specific $CD8^+$ T cells has been reported [43,44]. PCA analysis revealed that unlike IL-13Rα2 adjuvanted vaccine where IL-13 was transiently inhibited, complete lack of IL-13 (IL-13 KO group) not only increased RANTES, MIP-1β, and perforin mRNA expression by gut-$K^dGag_{197-205}$-specific $CD8^+$ T cells but also increased the levels of granzymes A and B mRNA expression. [Note that although the "number" of gut-$K^dGag_{197-205}$-specific single $CD8^+$ T cells that expressed granzymes A and B were lower in all four vaccination groups tested, the "level of expression" was significantly elevated in IL-13KO mice that received the control unadjuvanted vaccine, which is consistent with our previous findings [2].] It is now established that enhanced expression of perforin and granzymes by CD8 T cells correlate with enhanced cytotoxicity and vaccine-specific protective efficacy [30,45–47]. We have found that IL-13 KO mice given the control unadjuvanted vaccine were more protected against a surrogate mucosal influenza-HIV challenge compared to BALB/c mice given the IL-13Rα2 adjuvanted vaccine. This difference in protection in IL-13 KO mice could mainly be associated with the enhanced expression of RANTES, MIP-1β, perforin and also granzymes A and B by these $K^dGag_{197-205}$-specific $CD8^+$ T cells at the mucosae (enhanced expression of cytotoxic proteins). Interestingly, PCA revealed that the mean PC2 scores differed significantly between the IL-13 KO and the novel vaccine groups tested, highlighting the unique effects of transient inhibition of IL-13 verses total absence of IL-13 activity at the mucosae.

It is established that migration of lymphocytes to gut mucosae is mediated by integrin α4 heterodimerisation with β7 chain, not the β1 chain, and MAdCAM-1 ligand interacts preferentially with α4β7, not α4β1 [11,48], which is also consistent with our recent findings [68] Following the adjuvanted and unadjuvanted vaccination, relatively higher numbers of gut $K^dGag_{197-205}$-specific $CD8^+$ T cells were found to express β7 but not β1 mRNA, indicating that higher numbers of antigen-specific $CD8^+$ T cells could be trafficking to the gut mucosae. This is of importance in the context of an HIV vaccine, as the primary site of CD4 depletion is thought to be the gut mucosae [49,50]. In contrast, in all groups tested, increased numbers of β1 expressing splenic-$K^dGag_{197-205}$-specific $CD8^+$ T cells as well as elevated expression of β1 mRNA and protein were detected in splenic-$K^dGag_{197-205}$-specific compared to Peyer's patch, suggesting that in spleen integrin β1 preferentially heterodimerised with integrin α4 and not integrin β7. Interestingly, in $CD4^+$ T cells, elevated expression of β1 integrin subunit has shown to hamper integrin β7 interaction with integrin α4, resulting in suppression of α4β7 protein expression and T-cell homing to Peyer's patch [51]. Recently, using single-cell analysis, Kim et al. also have demonstrated that the co-engagement of α4β1 together with CD8/CD4 co-receptors on human T cells can also induce robust IL-2 and IFN-γ cytokine production [52]. Elevated IL-2 and IFN-γ expression by splenic-$K^dGag_{197-205}$-specific $CD8^+$ T cells (post HIV-peptide stimulation) have been detected in adjuvanted vaccinated group compared to mice given the unadjuvanted strategy [34,35]. These findings further indicate that integrin β1 may not only play a role in T-cell trafficking but also optimal T-cell activation and effector function.

In murine and non-human primate models, differential regulation of immune responses has been observed in systemic and mucosal compartments [37,53–56]. Consistent to other studies [57–59], our current data further confirm that there is a clear compartmentalization of immune responses between the spleen and gut compartments, even at the single cell level, highlighting the importance of evaluating both systemic and mucosal immunity, following mucosal vaccination. We believe that these differences are mainly linked to (i) the cytokine cell milieu induced following vaccination [2,68] and (ii) the resulting innate lymphoid cell subsets and antigen presenting cell subsets activated at the vaccination site [35,60], (iii) the mode of antigen uptake and presentation that take place via M-cells, which is exclusive to the mucosae [61,62], (iv) plus the homing signature acquired by the resulting T cells according to the anatomical location [68,63]. For example, it has been shown that priming with Peyer's patch derived DCs (but not splenic DCs) can induce the up-regulation of α4β7 and CCR9 on $CD8^+$ T cells and licence them to migrate to the gut mucosae [64]. In this study, although β7 mRNA and α4β7 protein expression levels were similar in splenic or Peyer's patch derived $K^dGag_{197-205}$-specific $CD8^+$ T cells, significantly elevated CCR9 expression was detected only in gut. Interestingly, PCA revealed a negative correlation of integrin α4 and CCR9 mRNA expression. MAdCAM-1 (which binds to α4β7) which is mainly detected in high endothelial venules of Peyer's patch is also thought to be present on

the sinus-lining cells in the spleen [65]. Thus, this may explain the unexpected abundance of $\alpha4\beta7^+$ $K^dGag_{197-205}$-specific splenic $CD8^+$ T cells observed, suggesting these may originate from gut (circulating gut T cells) [66]. In contrast, epithelial cells in the small intestine exclusively express the chemokine ligand 25 (CCL25), which can bind to CCR9 on T cells, thereby facilitating $CCR9^+$ T-cell homing to the intestine [67]. Thus, the current data indicated that compared to $\alpha4\beta7$, CCR9 is more specifically involved in gut-homing of HIV-specific $CD8^+$ T cells. Moreover, correlated expression of CCR9 and integrin αE was also established by PCA. Interestingly, expression of these two molecules was only detected in gut-$K^dGag_{197-205}$-specific single $CD8^+$ T cells, suggesting CCR9 and integrin αE could play a role in migration as well as retention of $CD8^+$ T cell in the intestinal mucosae [10]. It will be of interest to evaluate this homing marker expression at various mucosal effector sites.

Data indicate that where sample size is limited, single cell Fluidigm 48.48 Dynamic arrays can be successfully used to evaluate vaccine-specific mucosal immunity. The PCA demonstrated that novel IL-13Rα2 and IL-4R antagonist adjuvanted vaccines were uniquely different compared to the WT BALB/c control or IL-13KO groups given the unadjuvanted vaccine, indicating that level of IL-13 inhibition (total, transient or no inhibition) can significantly modulate the level of poly-functionality/cytotoxicity of $CD8^+$ T cells, altering the degree of T cell-mediated protective efficacy. The distinct clustering pattern of the spleen and gut $K^dGag_{197-205}$-specific single $CD8^+$ T cells, following PCA, also revealed that according to the anatomical location the mRNA expression profiles of the 21 genes tested can be significantly different, including Em and Cm T-cell ratios. Collectively, our analysis of the (i) relative numbers of cells expressing the mRNAs, (ii) poly-functionality in relation to RANTES expression and (iii) level of mRNA expression using PCA indicated that RANTES, MIP-1β, perforin and integrins α4, β1 and β7 could be used as a set of biomarkers to measure effective vaccine-specific mucosal immunity in HIV-specific single T cells obtained from mucosal or systemic compartments.

Author contributions

S.T. conducted all the experiments, a part of data analysis and prepared the manuscript, C.J. and T.N. performed the PCA and statistical analysis, R.R. developed and applied the Matlab code, R.J.J. designed and constructed all the rFPV, rVV, IL-13Ra2 and IL-4Rα antagonist adjuvanted vaccines, and C.R. helped design all the experiments and critical evaluation and preparation of the manuscript.

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Conflict of interest: The authors have no conflicts of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2015.10.085.

References

[1] Belyakov IM, Kuznetsov VA, Kelsall B, Klinman D, Moniuszko M, Lemon M, et al. Impact of vaccine-induced mucosal high-avidity $CD8^+$ CTLs in delay of AIDS viral dissemination from mucosa. Blood 2006;107:3258–64.
[2] Ranasinghe C, Turner SJ, McArthur C, Sutherland DB, Kim JH, Doherty PC, et al. Mucosal HIV-1 pox virus prime-boost immunization induces high-avidity $CD8^+$ T cells with regime-dependent cytokine/granzyme B profiles. J Immunol 2007;178:2370–9.
[3] Shacklett BL, Ferre AL. Mucosal immunity in HIV controllers: the right place at the right time. Curr Opin HIV AIDS 2011;6:202–7.
[4] Ferre AL, Hunt PW, Critchfield JW, Young DH, Morris MM, Garcia JC, et al. Mucosal immune responses to HIV-1 in elite controllers: a potential correlate of immune control. Blood 2009;113:3978–89.
[5] Huang X, Liu L, Ren L, Qiu C, Wan Y, Xu J. Mucosal priming with replicative *Tiantan vaccinia* and systemic boosting with DNA vaccine raised strong mucosal and systemic HIV-specific immune responses. Vaccine 2007;25:8874–84.
[6] Kaul D, Ogra PL. Mucosal responses to parenteral and mucosal vaccines. Dev Biol Stand 1998;95:141–6.
[7] Mestecky J. The common mucosal immune system and current strategies for induction of immune responses in external secretions. J Clin Immunol 1987;7:265–76.
[8] Hynes RO. Integrins: bidirectional, allosteric signaling machines. Cell 2002;110:673–87.
[9] Berlin C, Berg EL, Briskin MJ, Andrew DP, Kilshaw PJ, Holzmann B, et al. Alpha 4 beta 7 integrin mediates lymphocyte binding to the mucosal vascular addressin MAdCAM-1. Cell 1993;74:185–95.
[10] Cepek KL, Shaw SK, Parker CM, Russell GJ, Morrow JS, Rimm DL, et al. Adhesion between epithelial cells and T lymphocytes mediated by E-cadherin and the alpha E beta 7 integrin. Nature 1994;372:190–3.
[11] Hamann A, Andrew DP, Jablonski-Westrich D, Holzmann B, Butcher EC. Role of alpha 4-integrins in lymphocyte homing to mucosal tissues in vivo. J Immunol 1994;152:3282–93.
[12] Onai N, Kitabatake M, Zhang YY, Ishikawa H, Ishikawa S, Matsushima K. Pivotal role of CCL25 (TECK)-CCR9 in the formation of gut cryptopatches and consequent appearance of intestinal intraepithelial T lymphocytes. Int Immunol 2002;14:687–94.
[13] Wurbel MA, Malissen M, Guy-Grand D, Meffre E, Nussenzweig MC, Richelme M, et al. Mice lacking the CCR9 CC-chemokine receptor show a mild impairment of early T- and B-cell development and a reduction in T-cell receptor gammadelta(+) gut intraepithelial lymphocytes. Blood 2001;98:2626–32.
[14] Komoriya A, Green LJ, Mervic M, Yamada SS, Yamada KM, Humphries MJ. The minimal essential sequence for a major cell type-specific adhesion site (CS1) within the alternatively spliced type III connecting segment domain of fibronectin is leucine-aspartic acid-valine. J Biol Chem 1991;266:15075–9.
[15] Osborn L, Vassallo C, Browning BG, Tizard R, Haskard DO, Benjamin CD, et al. Arrangement of domains, and amino acid residues required for binding of vascular cell adhesion molecule-1 to its counter-receptor VLA-4 (alpha 4 beta 1). J Cell Biol 1994;124:601–8.
[16] Sixt M, Bauer M, Lammermann T, Fassler R. Beta1 integrins: zip codes and signaling relay for blood cells. Curr Opin Cell Biol 2006;18:482–90.
[17] Xu B, Wagner N, Pham LN, Magno V, Shan Z, Butcher EC, et al. Lymphocyte homing to bronchus-associated lymphoid tissue (BALT) is mediated by L-selectin/PNAd, alpha4beta1 integrin/VCAM-1, and LFA-1 adhesion pathways. J Exp Med 2003;197:1255–67.
[18] D'Souza MP, Altfeld M. Measuring HIV-1-specific T cell immunity: how valid are current assays. J Infect Dis 2008;197:337–9.
[19] Streeck H, Frahm N, Walker BD. The role of IFN-gamma Elispot assay in HIV vaccine research. Nat Protoc 2009;4:461–9.
[20] Varadarajan N, Julg B, Yamanaka YJ, Chen HB, Ogunniyi AO, McAndrew E, et al. A high-throughput single-cell analysis of human CD8(+) T cell functions reveals discordance for cytokine secretion and cytolysis. J Clin Invest 2011;121:4322–31.
[21] Loke P, Favre D, Hunt PW. Correlating cellular and molecular signatures of mucosal immunity that distinguish HIV controllers from noncontrollers (vol 115, pg e20, 2010). Blood 2010;116:2402.
[22] Ranasinghe C, Ramshaw IA. Immunisation route-dependent expression of IL-4/IL-13 can modulate HIV-specific CD8(+) CTL avidity. Eur J Immunol 2009;39:1819–30.
[23] Berger CT, Frahm N, Price DA, Mothe B, Ghebremichael M, Hartman KL, et al. High-functional-avidity cytotoxic T lymphocyte responses to HLA-B-restricted Gag-derived epitopes associated with relative HIV control. J Virol 2011;85:9334–45.
[24] Betts MR, Nason MC, West SM, De Rosa SC, Migueles SA, Abraham J, et al. HIV nonprogressors preferentially maintain highly functional HIV-specific $CD8^+$ T cells. Blood 2006;107:4781–9.

[25] Mothe B, Llano A, Ibarrondo J, Zamarreno J, Schiaulini M, Miranda C, et al. CTL responses of high functional avidity and broad variant cross-reactivity are associated with HIV control. PLoS ONE 2012;7:e29717.
[26] Ahmed RK, Makitalo B, Karlen K, Nilsson C, Biberfeld G, Thorstensson R. Spontaneous production of RANTES and antigen-specific IFN-gamma production in macaques vaccinated with SHIV-4 correlates with protection against SIVsm challenge. Clin Exp Immunol 2002;129:11–8.
[27] Cocchi F, DeVico AL, Garzino-Demo A, Arya SK, Gallo RC, Lusso P. Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells. Science 1995;270:1811–5.
[28] Ferbas J, Giorgi JV, Amini S, Grovit-Ferbas K, Wiley DJ, Detels R, et al. Antigen-specific production of RANTES, macrophage inflammatory protein (MIP)-1alpha, and MIP-1beta in vitro is a correlate of reduced human immunodeficiency virus burden in vivo. J Infect Dis 2000;182:1247–50.
[29] Hersperger AR, Pereyra F, Nason M, Demers K, Sheth P, Shin LY, et al. Perforin expression directly ex vivo by HIV-specific CD8 T-cells is a correlate of HIV elite control. PLoS Pathog 2010;6:e1000917.
[30] Migueles SA, Osborne CM, Royce C, Compton AA, Joshi RP, Weeks KA, et al. Lytic granule loading of CD8+ T cells is required for HIV-infected cell elimination associated with immune control. Immunity 2008;29:1009–21.
[31] Shankar P, Xu Z, Lieberman J. Viral-specific cytotoxic T lymphocytes lyse human immunodeficiency virus-infected primary T lymphocytes by the granule exocytosis pathway. Blood 1999;94:3084–93.
[32] Jespers V, Harandi AM, Hinkula J, Medaglini D, Le Grand R, Stahl-Hennig C, et al. Assessment of mucosal immunity to HIV-1. Expert Rev Vaccines 2010;9:381–94.
[33] Hamilton A, Harris G, Lee M, Pieprzyk M, Mir A, Livak K. BioMark dynamic arrays for single-cell gene expression analysis. J Stem Cells Regen Med 2010;6:87.
[34] Jackson RJ, Worley M, Trivedi S, Ranasinghe C. Novel HIV IL-4R antagonist vaccine strategy can induce both high avidity CD8 T and B cell immunity with greater protective efficacy. Vaccine 2014;20:1136–40.
[35] Ranasinghe C, Trivedi S, Stambas J, Jackson RJ. Unique IL-13Ralpha2-based HIV-1 vaccine strategy to enhance mucosal immunity, CD8(+) T-cell avidity and protective immunity. Mucosal Immunol 2013;6:1068–80.
[36] Xi Y, Day SL, Jackson RJ, Ranasinghe C. Role of novel type I interferon epsilon in viral infection and mucosal immunity. Mucosal Immunol 2012;5(6):610–22.
[37] Ranasinghe C, Medveczky JC, Woltring D, Gao K, Thomson S, Coupar BEH, et al. Evaluation of fowlpox-vaccinia virus prime-boost vaccine strategies for high-level mucosal and systemic Immunity against HIV-1. Vaccine 2006;24:5881–95.
[38] R Core Team. R: a language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2013. URL: http://www.R-project.org/.
[39] SPSS. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.; 2012.
[40] Matlab, MATLAB Version 8.0, The MathWorks, Inc., Natick, MA, United States.
[41] Kuerten S, Nowacki TM, Kleen TO, Asaad RJ, Lehmann PV, Tary-Lehmann M. Dissociated production of perforin, granzyme B, and IFN-gamma by HIV-specific CD8(+) cells in HIV infection. AIDS Res Hum Retrovir 2008;24:62–71.
[42] Crawford A, Angelosanto JM, Nadwodny KL, Blackburn SD, Wherry EJ. A role for the chemokine RANTES in regulating CD8 T cell responses during chronic viral infection. PLoS Pathog 2011;7:e1002098.
[43] Ullum H, Lepri AC, Victor J, Aladdin H, Phillips AN, Gerstoft J, et al. Production of beta-chemokines in human immunodeficiency virus (HIV) infection: evidence that high levels of macrophage in inflammatory protein-1 beta are associated with a decreased risk of HIV disease progression. J Infect Dis 1998;177:331–6.
[44] Migueles SA, Laborico AC, Shupert WL, Sabbaghian MS, Rabin R, Hallahan CW, et al. HIV-specific CD8+ T cell proliferation is coupled to perforin expression and is maintained in nonprogressors. Nat Immunol 2002;3:1061–8.
[45] Trapani JA, Smyth MJ. Functional significance of the perforin/granzyme cell death pathway. Nat Rev Immunol 2002;2:735–47.
[46] Chattopadhyay PK, Betts MR, Price DA, Gostick E, Horton H, Roederer M, et al. The cytolytic enzymes granyzme A, granzyme B, and perforin: expression patterns, cell distribution, and their relationship to cell maturity and bright CD57 expression. J Leukoc Biol 2009;85:88–97.
[47] Harari A, Bellutti Enders F, Cellerai C, Bart PA, Pantaleo G. Distinct profiles of cytotoxic granules in memory CD8 T cells correlate with function, differentiation stage, and antigen exposure. J Virol 2009;83:2862–71.
[48] Kilshaw PJ, Murant SJ. Expression and regulation of beta 7(beta p) integrins on mouse lymphocytes: relevance to the mucosal immune system. Eur J Immunol 1991;21:2591–7.
[49] Holmgren J, Czerkinsky C. Mucosal immunity and vaccines. Nat Med 2005;11:S45–53.
[50] Neutra MR, Kozlowski PA. Mucosal vaccines: the promise and the challenge. Nat Rev Immunol 2006;6:148–58.
[51] DeNucci CC, Pagan AJ, Mitchell JS, Shimizu Y. Control of alpha4beta7 integrin expression and CD4 T cell homing by the beta1 integrin subunit. J Immunol 2010;184:2458–67.
[52] Kim TK, Billard MJ, Wieder ED, McIntyre BW, Komanduri KV. Co-engagement of alpha(4)beta(1) integrin (VLA-4) and CD4 or CD8 is necessary to induce maximal Erk1/2 phosphorylation and cytokine production in human T cells. Hum Immunol 2010;71:23–8.
[53] Belyakov IM, Isakov D, Zhu Q, Dzutsev A, Berzofsky JA. A novel functional CTL avidity/activity compartmentalization to the site of mucosal immunization contributes to protection of macaques against simian/human immunodeficiency viral depletion of mucosal CD4+ T cells. J Immunol 2007;178:7211–21.
[54] Yang OO, Ibarrondo FJ, Price C, Hultin LE, Elliott J, Hultin PM, et al. Differential blood and mucosal immune responses against an HIV-1 vaccine administered via inguinal or deltoid injection. PLOS ONE 2014;9:e88621.
[55] Kent SJ, Dale CJ, Ranasinghe C, Stratov I, De Rose R, Chea S, et al. Mucosally-administered human-simian immunodeficiency virus DNA and fowlpoxvirus-based recombinant vaccines reduce acute phase viral replication in macaques following vaginal challenge with CCR5-tropic SHIVSF162P3. Vaccine 2005;23:5009–21.
[56] Ranasinghe C, Eyers F, Stambas J, Boyle DB, Ramshaw IA, Ramsay AJ. A comparative analysis of HIV-specific mucosal/systemic T cell immunity and avidity following rDNA/rFPV and poxvirus-poxvirus prime boost immunisations. Vaccine 2011;29:3008–20.
[57] Masopust D, Vezys V, Wherry EJ, Barber DL, Ahmed R. Cutting edge: gut microenvironment promotes differentiation of a unique memory CD8 T cell population. J Immunol 2006;176:2079–83.
[58] Klonowski KD, Williams KJ, Marzo AL, Blair DA, Lingenheld EG, Lefrancois L. Dynamics of blood-borne CD8 memory T cell migration in vivo. Immunity 2004;20:551–62.
[59] Wang XL, Xu HB, Alvarez X, Pahar B, Moroney-Rasmussen T, Lackner AA, et al. Distinct expression patterns of CD69 in mucosal and systemic lymphoid tissues in primary SIV infection of rhesus macaques. PLoS ONE 2011;6.
[60] Trivedi S, Jackson RJ, Ranasinghe C. Different HIV pox viral vector-based vaccines and adjuvants can induce unique antigen presenting cells that modulate CD8 T cell avidity. Virology 2014;468–470:479–89.
[61] Hase K, Kawano K, Nochi T, Pontes GS, Fukuda S, Ebisawa M, et al. Uptake through glycoprotein 2 of FimH(+) bacteria by M cells initiates mucosal immune response. Nature 2009;462:226–30.
[62] Kiyono H, Fukuyama S. NALT- versus peyer's-patch mediated mucosal immunity. Nat Immunol 2004;4:699–710.
[63] Kaufman DR, Barouch DH. Translational mini-review series on vaccines for HIV: T lymphocyte trafficking and vaccine-elicited mucosal immunity. Clin Exp Immunol 2009;157:165–73.
[64] Mora JR, Bono MR, Manjunath N, Weninger W, Cavanagh LL, Rosemblatt M, et al. Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells. Nature 2003;424:88–93.
[65] Kraal G, Schornagel K, Streeter PR, Holzmann B, Butcher EC. Expression of the mucosal vascular addressin, MAdCAM-1, on sinus-lining cells in the spleen. Am J Pathol 1995;147:763–71.
[66] Erle DJ, Briskin MJ, Butcher EC, Garcia-Pardo A, Lazarovits AI, Tidswell M. Expression and function of the MAdCAM-1 receptor, integrin alpha 4 beta 7, on human leukocytes. J Immunol 1994;153:517–28.
[67] Svensson M, Marsal J, Ericsson A, Carramolino L, Broden T, Marquez G, et al. CCL25 mediates the localization of recently activated CD8alphabeta(+) lymphocytes to the small-intestinal mucosa. J Clin Invest 2002;110:1113–21.
[68] Trivedi S, Ranasighe C. The influence of immunization route, tissue microenvironment, and cytokine cell milieu on HIV-specific CD8+ T cells measured using Fluidigm Dynamic arrays. PLOS ONE 2015;10(5):e0126487, http://dx.doi.org/10.1371/journal.pone.0126487.